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Research Paper

Role of Natural Autoantibodies in Ugandans With Rheumatic Heart Disease and HIV☆

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ABSTRACT

Background: Rheumatic heart disease (RHD) and HIV are prevalent diseases in sub-Saharan Africa, but little is known about their potential interrelationships. The objective of this study was to assess the prevalence of protective natural autoantibodies among patients with RHD in Uganda, and to determine whether the levels of these autoantibodies are affected by HIV status.

Methods: Participants were grouped according to RHD and HIV status. The three control groups (RHD − HIV −, RHD − HIV +, RHD + HIV −) were age-matched to the RHD + HIV + participants. All participants underwent HIV testing and echocardiography to evaluate for RHD. Natural autoantibody levels reactive with phosphorylcholine (PC) and malondialdehyde (MDA) were measured.

Findings: We enrolled 220 participants; 21 with both RHD and HIV. Ages ranged from 10 to 60 years, with female predominance (144/220, 65%). After adjusting for age and gender, HIV infection and RHD were each associated with low IgM anti-PC (HIV: $p < 0.0001$ and RHD: $p = 0.01$). A distinct HIV∗RHD interaction was identified ($p = 0.045$) with increased IgG anti-MDA levels in HIV infected subjects without RHD, whereas IgG anti-MDA levels were decreased in HIV infected subjects with RHD.

Interpretation: We found that HIV and RHD are associated with alterations in natural autoantibody responses previously linked to an increased risk for atherosclerosis and autoimmune inflammatory disease.

1. Introduction

Rheumatic heart disease (RHD) and HIV are prevalent in Uganda and other sub-Saharan African countries. Rheumatic heart disease (RHD) affects 15 million people worldwide with an estimated 1.4 million deaths annually. In Uganda RHD is the most common cause of heart disease within the 15 to 49 age group (Remenyi et al., 2012; Okello et al., 2012; Marijan et al., 2012; Sliwa and Zilla, 2012). The immunopathogenesis of chronic valvular inflammation in RHD including the role of cellular vs. humoral immune responses, and the identity of the responsible immunodominant epitopes that drive the progression of disease are debated (Tandon et al., 2013). HIV currently affects 5–10% of Ugandan adults, which is improved from nearly 30% in the early 1990s (The Republic of Uganda, 2014). The immune deficiency and dysfunction caused by HIV and AIDS has been shown to impact susceptibility to and progression of other autoimmune diseases (Zandman-Goddard and Shoenfeld, 2002); however, the impact of HIV infection on RHD pathogenesis has not been investigated.

Natural arising autoantibodies to oxidation-associated epitopes have been shown to modulate the initiation and progression of a range of immune-mediated inflammatory diseases including atherosclerosis. These autoantibodies are postulated to play roles in homeostasis and immune regulation (Gronwall and Silverman, 2014), and altered serum levels correlate with certain disease states. IgM natural autoantibodies reactive with the phosphorylcholine (PC) head group are commonly cross-reactive with PC determinants on oxidatively modified

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low density lipoprotein (LDL), and apoptotic cells but not with healthy cells. In mice and humans, the antibody genes for dominant B-cell clones to PC determinants show evidence of clonal restriction and convergent somatic evolution (Silverman, 2015). Malondialdehyde (MDA) is a small chemically reactive compound that readily forms adducts on proteins, and IgM anti-MDA antibodies are common at birth and show great structural and clonal diversity (Chou et al., 2009; Unpublished data). Induction of high levels of IgM anti-PC antibodies has been shown to protect hyperlipidemic mice from atherosclerosis (Binder et al., 2003), and low IgM anti-PC correlates with increased risk for stroke and myocardial infarction in population-based cohort studies (Fiskesund et al., 2010; Gronlund et al., 2009). These natural autoantibodies may also modulate the pathogenesis of experimental and clinical autoimmune disease. IgM antibodies to PC enhance the clearance of damaged and apoptotic cells by binding to oxidation-associated epitopes, enhancing recruitment of C1q and mannose binding lectin and suppressing Toll-like receptor mediated inflammatory responses (Chen et al., 2009a; Chen et al., 2009b; Vas et al., 2012). In systemic lupus erythematous (SLE) patients, higher levels of anti-PC IgM have been associated with lower clinical disease activity, lower rates of MI and stroke, as well as reduced preclinical atherosclerosis burden based on carotid ultrasound (Gronwall et al., 2012; Gronwall et al., 2014).

Due to the common expression of these natural autoantibodies, we postulated that there may be similar roles in resolution of cellular injury in damaged valvular tissues in RHD, yet this has not previously been studied. In theory, high IgM anti-PC antibody titers would be predicted to promote rapid clearance of apoptotic cells in damaged tissues, suppressing over-exuberant inflammatory responses and release of autoantigens from cells, which may also have direct or indirect influence on recruitment and activation of auto-reactive lymphocytes. Given their putative role(s) in inflammatory vascular conditions, protective natural autoantibodies may also affect the risk of atherosclerosis in patients with RHD and HIV. The chronic systemic inflammation that is a hallmark of RHD and rheumatic fever may contribute to increased risk of atherosclerosis (Habeeb and Hadidi, 2011; Zoller et al., 2012). HIV infection is a well-known risk factor for accelerated atherosclerosis independent of use of antiretroviral therapy (ART), due to chronic inflammation and immune activation (Longenecker and Triant, 2014). In a recent study, HIV infection was associated with lower protective IgM natural autoantibodies and higher levels of IgG natural autoantibodies against oxidized forms of LDL (Yilmaz et al., 2014) but the relevance to clinical cardiovascular disease was not examined (Tsimikas et al., 2007). Perturbations in the levels of these natural autoantibodies may therefore contribute to the accelerated atherosclerosis seen in HIV.

The purpose of the current study was to assess the relative prevalence of these natural autoantibodies among patients with and without RHD in Uganda and to determine whether the presence of these autoantibodies may be impacted by HIV infection. We sought to test the hypothesis that both conditions would be associated with reduced levels of oxidation-associated IgM anti-PC natural autoantibodies along with increased IgG anti-MDA antibodies.

2. Methods

2.1. Study Participants and Enrollment

Study participants were enrolled from the Joint Clinical Research Centre and the Uganda Heart Institute in Kampala, Uganda from April to June 2014. For this cross-sectional study, we recruited subjects that were then stratified by RHD and HIV status into groups (RHD − HIV −, RHD − HIV +, RHD + HIV −) that were age-matched to the RHD + HIV + group. All participants were >8 years old and had a transthoracic echocardiogram performed for valvular heart disease, due to clinical presentation or as part of a screening protocol. We included those with overt clinical RHD or definite latent RHD, as defined by World Heart Federation (WHF) criteria (Remenyi et al., 2012). Subjects were excluded from the RHD negative controls if they had elevated anti-streptolysin O, borderline RHD by WHF criteria or had congenital heart disease or heart failure other than from RHD. Written informed consent was obtained from each participant and the study was approved by the Institutional Review Boards at University Hospitals, Cleveland, Ohio and Makerere University, Kampala, Uganda.

HIV-1/2 testing was performed on all participants, unless an HIV diagnosis had previously been confirmed with rapid antibody testing and confirmed by ELISA or Western Blot. We used a primary rapid antibody test (Alere Determine HIV-1/2) with confirmation by Trinity Biotech Uni-Gold and AccuBioTech tests. Patients who were newly HIV positive were also referred for further care and confirmatory testing to the Joint Clinical Research Centre or other preferred HIV treatment center. Venous blood was obtained by venipuncture and a complete blood count, Anti-streptolysin O (ASO), and high sensitivity CRP (hsCRP) were measured at the clinical laboratory of the Uganda Heart Institute. CD4 count was measured in all HIV positive subjects. Serum samples were frozen and stored at the Joint Clinical Research Centre at −80 °C without thawing before autoantibody analysis. At the time of enrollment clinical history was obtained by chart review. All data were stored in a REDCap database hosted at University Hospitals, Cleveland (Harris et al., 2009).

2.2. Natural Autoantibodies

Levels of anti-phosphorylcholine (PC) and anti-malondialdeyhyde (MDA) antibodies were measured in duplicate from frozen serum samples by in-house assays, as previously described (Gronwall et al., 2012). Herein, ELISA wells were coated with PC6-BSA (Biosearch Technologies Inc., Novato, CA, USA) or MDA-BSA (Academic Bio-medical Co., Houston, TX, USA). Values were reported from 1:1000 dilutions in 1% BSA-PBS after detection with goat anti-IgM-HRP (Southern Biotech, Birmingham, AL, USA) or goat anti-IgG-HRP (Jackson Immunosearch, West Grove, PA, USA). An established standard curve from a SLE pool was used for calibration. Assays for IgG anti-PC or IgM anti-MDA were not included as pilot studies have shown these levels to be non-informative [data not shown] (Gronwall et al., 2012).

2.3. Statistics

Baseline demographic and clinical characteristics were described with standard descriptive statistics. Median natural autoantibody or inflammatory marker levels were compared between groups using the Kruskal–Wallis test. Linear regression was used to assess the effect of HIV and RHD on autoantibody and inflammatory marker levels while adjusting for age and sex and accounting for potential effect modification between HIV and RHD by testing their cross product term. The linearity and normality of residuals assumptions were assessed with residual by predicted plots and quantile–quantile plots respectively. Violations were rectified by log-transforming the outcome variables. P values less than 0.05 were considered significant. SAS 9.3 was used for statistical analyses (SAS Institute Inc., Cary, NC, USA).

2.4. Funding

The study was funded by the National Institutes of Health, American College of Rheumatology, and Medtronic Philanthropy. No funding source had any role in the design of the study.

3. Results

Overall, 231 participants were enrolled at the two Ugandan sites (Fig. 1); however, eleven were later excluded from the RHD negative control group because of an elevated ASO or an abnormal screening

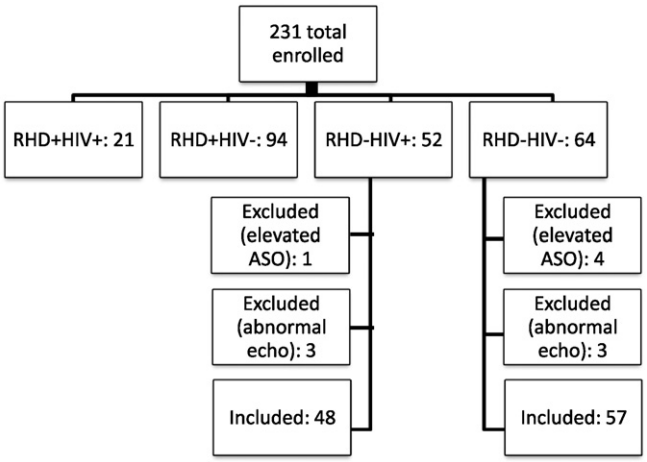

Fig. 1. Total enrollment at the Uganda Heart Institute and Joint Clinical Research Centre.

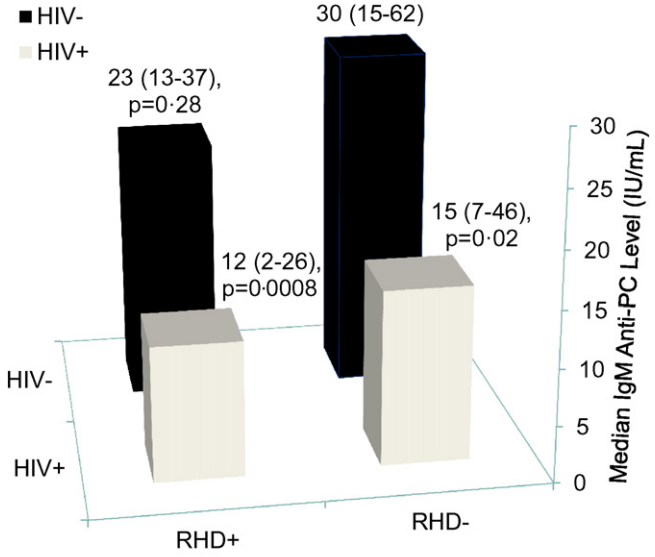

Fig. 2. Median (IQR) levels of protective IgM anti-PC by RHD and HIV status.

echocardiogram. The baseline characteristics of the final 220 study participants are presented in Table 1. Age distribution was similar between groups by design. CD4 counts and rates of ART use among HIV groups were similar ($p > 0.71$). Participants with RHD (RHD + HIV − and RHD + HIV +) had higher levels of hsCRP ($p < 0.0001$). The distribution of aortic/mitral regurgitation and stenosis was similar among the two RHD groups ($p > 0.43$).

The median anti-PC and anti-MDA levels by group, comparing each disease group with the RHD − HIV − controls, are depicted in Figs. 2 and 3. We found that anti-PC IgM was lower in both HIV + groups compared to the RHD − HIV − group, while for anti-MDA IgG there were no significant differences between the 3 disease groups and controls (all $p > 0.10$). In further subgroup analysis of HIV positive individuals, those with CD4 count <200 ($n = 12$) did not differ with respect to anti-PC IgM when compared to those with a CD4 count >500 ($n = 33$) [median (Q_{25}–Q_{75}): 12 (8–40) U/mL vs. 19 (7–37) U/mL, $p = 0{\cdot}91$]. Similarly, there was no difference in anti-MDA IgG [16 (7–25) U/mL for CD4 < 200 vs. 11 (3–25) U/mL for CD4 > 500, $p = 0.64$].

To further explore the effect of HIV and RHD on autoantibody levels, an adjusted analysis allowing for interaction was conducted (Table 2). In these models, HIV infection and RHD were each independently associated with lower anti-PC IgM (HIV: $p < 0.0001$ and RHD: $p = 0.012$). There was no evidence of effect modification interaction between RHD

Table 1
Baseline characteristics of study participants.

Characteristics	Total	RHD − HIV −	RHD + HIV +	RHD + HIV −	RHD − HIV +
Count	220	57	21	94	48
Age (years)[a]	33 (11)	33 (11)	33 (11)	33 (11)	32 (12)
Age range	10–60	15–60	13–52	13–53	10–52
Female	144 (65)	32 (56)	17 (81)	61 (65)	34 (71)
Baseline lab values					
WBC[b]	4.8 (3.7–5.9)	4.8 (3.7–5.9)	4.1 (3.7–4.9)	5.6 (4.5–6.6)	3.9 (3.1–4.7)
Leukopenia (<4)	65 (30)	19 (33)	7 (33)	15 (16)	24 (50)
HGB[b]	13.9 (12.7–15.5)	14.5 (13.1–15.8)	13.5 (12.5–15.1)	14.0 (12.6–15.8)	13.3 (12.3–14·4)
Anemia (<12.1)	34 (15)	8 (14)	3 (14)	15 (16)	8 (17)
PLT[b]	241 (187–290)	242 (179–274)	245 (218–287)	219 (179–281)	270 (219–353)
Thrombocytopenia	21 (10)	7 (12)	1 (5)	12 (13)	1 (2)
ASO[b]	61 (38–96)	61 (43–82)	41 (20–125)	68 (45–104)	38 (22–67)
>200	6 (3)	0	1 (0)	5 (5)	0
hsCRP[b]	2.28 (0.67–5.72)	1.15 (0.47–2.95)	2.12 (0.80–4.09)	3.99 (1.64–12.37)	1.21 (0.43–4.62)
0–1	75 (34)	28 (49)	7 (33)	18 (19)	21 (44)
1–3	55 (25)	15 (26)	5 (24)	22 (23)	13 (27)
>3	89 (41)	14 (25)	9 (43)	54 (57)	14 (29)
RHD characteristics					
Mitral or aortic disease rate					
Mitral regurgitation	N/A	0 (0)	17 (81)	66 (70)	0 (0)
Mitral stenosis	N/A	0 (0)	8 (38)	42 (45)	0 (0)
Aortic regurgitation	N/A	0 (0)	7 (33)	43 (46)	0 (0)
Aortic stenosis	N/A	0 (0)	1 (5)	8 (8)	0 (0)
Ejection fraction <55%	N/A	1 (2)	2 (10)	18 (19)	0 (0)
HIV characteristics					
CD4[b]	N/A	N/A	493 (364–816)	N/A	536 (317–623)
Diagnosis CD4[b]	N/A	N/A	237 (39–680)	N/A	435 (137–648)
HIV diagnosis duration (years)[b]	N/A	N/A	9 (4–11)	N/A	6 (5–9)
On ART	N/A	N/A	19 (90)	N/A	42 (88)
ART duration (years)[b]	N/A	N/A	6 (2–10)	N/A	6 (1–8)

[a] Mean (SD).
[b] Median (IQR).

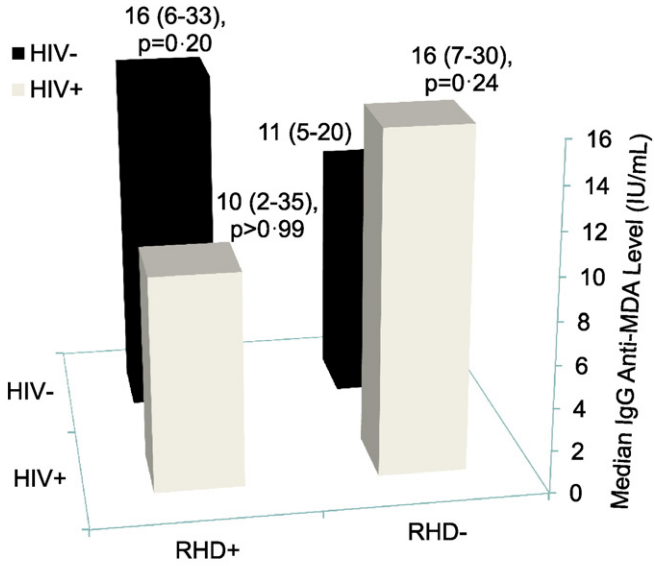

Fig. 3. Median (IQR) levels of IgG anti-MDA by RHD and HIV status.

and HIV ($p = 0.50$) for levels of anti-PC IgM, and thus it was removed from the model. A significant HIV*RHD interaction was identified for IgG anti-MDA ($p = 0.045$) such that in participants without RHD we found that HIV infection was associated with increased IgG anti-MDA, whereas it was associated with decreased IgG anti-MDA in participants with RHD.

4. Discussion

Our study demonstrates that HIV infection is associated with low levels of IgM natural autoantibody to the oxidation-associated epitope phosphorylcholine (PC), and high IgG natural autoantibody to the distinct oxidation associated epitope, malondialdehyde (MDA). Phosphorylcholine and malondialdehyde containing determinants are both commonly expressed on apoptotic cells and on oxidized LDL that is prevalent in atherosclerotic plaques. Studies in other conditions have supported the notion that IgM natural autoantibodies to PC can protect against autoimmune diseases and atherosclerosis, whereas IgG anti-MDA antibodies are associated with more severe autoimmune disease and higher risk of atherosclerotic cardiovascular disease.

These relationships between autoantibody responses and disease have been well characterized in patients with SLE, the archetypic systemic autoimmune disease (Gronwall et al., 2012); however, low IgM anti-PC has also been associated with atherosclerosis and increased risk of myocardial infarction and stroke in populations without known autoimmune disease (Fiskesund et al., 2010; Gronlund et al., 2009). In these latter studies, anti-PC IgM titer below the 30th percentile corresponded with a multivariable adjusted relative risk of 1.62 (CI 1.11–2.35) for stroke, while a level below the 25th percentile was associated with a relative risk of 1.69 (1.09–2.54) for myocardial infarction. In our study, half of participants with HIV and over two-thirds of those with both RHD and HIV had IgM anti-PC levels below the 25th percentile of the RHD and HIV negative group (Supplementary Figs. 1 and 2), suggesting that the degree to which these natural autoantibodies are altered in HIV infection may also be associated with a clinically significant increase in myocardial infarction and stroke. While we did not have statistical power to determine whether very low CD4 T-cell count in HIV patients was associated with altered levels of autoantibodies, given the associations in other cohorts between low nadir and proximal CD4+ T-cells counts and cardiovascular events (Longenecker and Triant, 2014), this should be further explored in future studies.

Table 2
HIV and RHD are independently associated with low IgM anti-PC in multivariable linear regression models of autoantibody levels.

	IgM anti-PC[a]		IgG anti-MDA[a]	
Exposure	β	P	β	P
Age (year)	−0.005	0.12	−0.009	0.012
Female	0.074	0.31	−0.158	0.048
HIV+	−0.341	<0.0001	0.201	0.75
RHD+	−0.182	0.012	0.133	0.64
HIV+ RHD+	–	–	−0.347	0.045

[a] Log 10 transformed.

Anti-MDA IgG has previously been associated with higher SLE disease activity (Gronwall et al., 2012). In addition, higher MDA-LDL levels in immune complexes have previously been associated with increased rates of myocardial infarction (Lopes-Virella et al., 2012). Higher levels of circulating IgG—especially the proinflammatory IgG1 and IgG3 subclasses that activate Fcγ receptors and complement—against oxidation-associated epitopes (on LDL or other macromolecules) may occur in the context of a higher burden of inflammatory atherosclerotic plaque (Mironova et al., 1996). Indeed we observed higher IgG against MDA in individuals with HIV, which we postulate may correspond to increased inflammation and formation of oxidation byproducts in these patients.

Although these specific types of natural autoantibodies that were the focus of our investigations have not been previously studied in the context of HIV, in a recent study, levels of anti-MDA-LDL antibodies were evaluated in HIV-infected and uninfected subjects. It found lower levels of IgM natural autoantibody against MDA-LDL and higher levels of IgG against MDA-LDL, although further molecular characterizations were not performed (Yilmaz et al., 2014). Another study of IgG antibodies to oxidized LDL in HIV patients found a decrease in the levels of these antibodies after immune reconstitution following initiation of antiretroviral therapy (Ronchini et al., 2013). Our findings are therefore similar to patterns previously reported in HIV infected subjects.

We found evidence of similar alterations in levels of certain natural autoantibodies (low IgM anti-PC and high IgG anti-MDA) among patients with RHD, although the effect size was smaller than for HIV infection. As an autoimmune condition, RHD may be associated with global immune dysregulation that also affects levels of these natural autoantibodies, and further study with larger sample sizes are needed to more clearly reveal this association. For the reasons mentioned above, these findings may also provide insight into atherosclerotic vascular disease risk in patients with RHD. Compared to classical atherosclerotic cardiovascular disease, patients with RHD tend to be younger and their cardiovascular morbidity and mortality are driven more by complications of progressive valvular heart failure and the need for heart valve surgery. However, a national database study from Sweden reported that hospitalization for acute rheumatic fever was associated with a 4.7-fold increased risk of subsequent coronary heart disease (Zoller et al., 2012). While intriguing, this finding could potentially be confounded by ascertainment bias (i.e. RHD patients undergoing heart valve surgery are routinely evaluated for co-existing coronary artery disease). Another study found that carotid intima-media thickness and aortic stiffness were increased in children with a prior diagnosis of rheumatic fever (Ciftel et al., 2014). Therefore the relationships between natural autoantibodies to oxidation-associated antigens, subclinical vascular disease, ventricular-vascular interactions, and RHD disease susceptibility and progression should be evaluated in future studies.

A central aim of our study was to examine the interaction between RHD and HIV status—two highly prevalent diseases in sub-Saharan Africa. Very little is known about this interaction, although there is a reason to believe that concurrent HIV infection may alter susceptibility

to RHD or RHD disease progression in positive and negative ways (Longenecker et al., 2014). Our group has previously reported a decreased prevalence of latent rheumatic heart disease detected by echocardiography among HIV-infected children compared to other Ugandan school children (Gleason et al., 2014) but there are many health system factors (e.g. access to healthcare services, use of prophylactic antibiotics) that may confound this finding. Besides the finding that HIV infection may adversely affect the natural autoantibody responses, other findings from our study suggest that HIV-infected patients with RHD may be different from HIV-uninfected patients with RHD in ways that may protect against RHD progression. First, there was an interaction between RHD and HIV in our multivariable model, such that HIV infection was associated with *lower* anti-MDA IgG levels among subjects with RHD—this is opposite of the effect of HIV in the overall cohort. Second, HIV-infected subjects with and without RHD had lower ASO titers than HIV-uninfected controls. This may reflect a decreased burden of invasive GAS infection among subjects with HIV, possibly due to better engagement in care and access to antibiotics. Compared to uninfected subjects with RHD, HIV-infected subjects with RHD had lower levels of systemic inflammation as measured by hsCRP, again suggesting some protective confounder.

Among the first reported immune abnormality in HIV infected individuals was the appearance of rheumatoid factors and anti-nuclear antibodies (Lane et al., 1985), and HIV infection also has been reported to impact risk for some autoimmune diseases (Zandman-Goddard and Shoenfeld, 2002). The immunosuppression caused by HIV at low CD4 + T-cell counts may protect against autoimmune disease; in fact, patients with SLE have been reported to symptomatically improve as HIV disease progresses and CD4 counts declines, but then may later symptomatically worsen after immune reconstitution with ART (Zandman-Goddard and Shoenfeld, 2002). In the era of effective ART, autoimmune disease may occur as an immune reconstitution inflammatory syndrome (IRIS), but overall the incidence of associated connective tissue diseases appears to be on the decline (Calabrese et al., 2005; Iordache et al., 2014). In our study, HIV infection was associated with perturbations in levels of natural autoantibodies to oxidation-associated determinants that have been previously associated with increased disease severity in SLE (Gronwall et al., 2012). HIV infection likely has a complex relationship with autoimmune predisposition to diseases such as SLE and to RHD — perturbations in these types of natural autoantibodies may also increase risk and disease severity while the immunosuppression and profound CD4 T cell depletion in untreated HIV may reduce risk and disease severity.

Our study has limitations. As with all cross-sectional studies, ours cannot prove causal relationships or account for all potential confounders. Furthermore, our study may have been underpowered to detect a significant effect of CD4 count on natural autoantibodies. Because our study was limited to Ugandans, our findings may not be applicable to other populations that also have a high prevalence of both HIV and RHD. Our study included patients with chronic RHD, but it would also be useful to study subjects with acute rheumatic fever. Future studies should also assess acquired autoantibodies that have been implicated in RHD pathogenesis, including antibodies against myosin, collagen, laminin, and other streptococcal proteins.

In conclusion, HIV infection appears to alter natural antibody levels in ways that may increase risk of atherosclerosis and may impact the pathogenesis of RHD. To our knowledge, there has been no prior study that has characterized these clinical and immune characteristics of HIV-infected patients with RHD, two immune disorders that are highly prevalent in sub-Saharan Africa. Both RHD and HIV appear to be associated with altered levels of circulating natural autoantibody to oxidation-associated determinants and therefore our findings suggest that there may be important interrelationships between these two clinical conditions. These findings may therefore have important implications for the study of autoimmunity as a contributing mechanism for cardiovascular disease in sub-Saharan Africa and in areas of high HIV-prevalence worldwide.

Sources of Funding/Conflicts of Interest

This study was funded by grants from the R.J. Fasenmeyer Center for Clinical Immunology at the Cleveland Clinic, Case Western Reserve University Rottman Fund, ASTMH Benjamin H. Kean Fellowship, IDSA Medical Scholars Program, the NIH-NIAID R01 AI090118, R01 A1068063, the American College of Rheumatology Research and Education Foundation Within Our Reach campaign, the National Institutes of Health (K23 HL123341), a Wolf Family Foundation Scholars Grant, and Medtronic Philanthropy. All natural autoantibody assays were performed at NYU. Funding sources did not have any role in the design of implementation of this study.

The authors do not have any other disclosures or conflicts of interests.

Author Contributions

DMH designed the study, supervised enrollment, wrote the analysis plan, analyzed the data, and drafted and revised the paper. EO designed the study, supervised enrollment, wrote the analysis plan, and revised the draft paper. GM supervised enrollment, and revised the draft paper. IS supervised enrollment and revised the draft paper. DAZ designed the study and revised the draft paper. GJS advised on the analysis plan, performed the natural autoantibody assays and revised the paper. LG advised on the analysis plan, performed the natural autoantibody assays and revised the draft paper. ASN advised on the statistical approaches of the analysis plan and revised the draft paper. LHN designed the study and revised the draft paper. RAS designed the study, wrote the analysis plan, and revised the draft paper. CTL designed the study, wrote the analysis plan, and revised the draft paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.02.006.

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